REMARKS

Claims 94-96 have been withdrawn from consideration.

Claims 72-93 have been examined on the merits.

Claims 72 and 92 have been amended. Claim 91 has been cancelled.

Thus, claims 72-90 and 92-96 are in the application.

Reexamination and reconsideration of the present claims in view of the remarks presented below are respectfully requested.

Restriction Requirement

Applicants note the Examiner's comments on page 2 of the outstanding Office Action that election of SEQ ID NO: 81 was in response to a Restriction of Group I.

Applicants also acknowledge that Claims 94-96 and sequences other than SEQ ID NO: 81 are withdrawn from further consideration.

Priority

Applicants respectfully direct the Examiner's attention to page 2 of the Preliminary Amendment filed on December 2, 2005, wherein it is indicate that present application is a United States national phase entry, pursuant to 35 U.S.C. 371, of PCT/US2004/017490, filed June 3, 2004.

Rejection of Claims 72-93 Under 35 U.S.C § 103(a)

Claims 72-93 stand rejected under 35 U.S.C § 103(a) as being unpatentable over Bennett et al. (U.S. Patent No. 6,335,194) or Bennett et al. (U.S. Patent No. 6,838,283) or Bennett et al. (U.S. Patent No. 7,288,530) or Bennett et al. (U.S. Patent No. 6,077,709) and Tuschl et al. (U.S. 2004/0259247) and Vickers et al. (The Journal of Biological Chemistry Vol. 278(9):7108-7118, 2003) and Morrissey et al. (U.S. 2003/0206887) and Arnold et al. (U.S. 6060456)

This rejection is respectfully traversed. Reconsideration and withdrawal thereof are requested.

The rejection asserts that it would have been *prima facie* obvious to one of ordinary skill in the art to use the presently claimed siRNA compound to target the same region in Survivin mRNA as an antisense compound taught by the Bennett el al. patents.

For the reasons set forth in detail below, Applicants respectfully submit that one of ordinary skill in the art would not have been motivated to use an siRNA to target the specific region of Survivin mRNA targeted by the antisense compounds disclosed in the Bennett et al. patents in view of the poor inhibitory activity of the latter, and that one would not have expected to achieve the high level of inhibition of Survivin expression obtained with the siRNA of SEQ ID NO:81 as in the present application. The Bennett et al. patents provide no reasonable expectation of success with respect to the present invention, and the results obtained with the siRNA of SEQ ID NO:81 are superior and unexpected, obviating *prima facie* obviousness.

Antisense compound sequences 24, 33, and 73 in the Bennett et al. patents target a poorly accessible region in Survivin mRNA

Applicants respectfully submit that this rejection is improper as the antisense compounds relied on in the Bennett et al. patents (SEQ ID NO:24 of U.S. Patent No. 6,335,194 and SEQ ID NOs:33 and 73 of the remaining Bennett patents) fail to provide motivation to design a corresponding siRNA. The Bennett et al. patents disclose that the antisense compounds noted above exhibit 2-8% inhibition of human Survivin. Note Tables 1 and 2 of U.S. Patent No. 6,077,709; U.S. Patent No. 6,838,283; U.S. Patent No. 7,288,530; and U.S. Patent No. 6,335,194. In view of these results, one of ordinary skill in the art would not have been motivated to target the same region of Survivin mRNA with an siRNA compound in view of the low inhibitory activity of the cited antisense compounds, presumably due to the inaccessibility of the target of the antisense compounds.

The rejection asserts on page 5, lines 5-7, of the outstanding Office Action, that "if an antisense is functional for a specific target one can have a reasonable expectation that an siRNA targeted to the same target will also be functional." In the present case, the antisense compound exhibits poor inhibitory activity. Consequently, one would also expect an siRNA targeting the same region in Survivin mRNA to have poor inhibitory activity, presumably due to inaccessibility of the target. Surprisingly, and in direct contrast, the siRNA of SEQ ID NO:81 of the present invention exhibits high inhibitory activity. Note, for example, the data at page 81,

table 5; page 86, tables 8 and 9; page 87, table 10; pages 88-89, table 11; and pages 89-90, table 12.

As noted by Branch (*A good antisense molecule is hard to find*, TIBS (1998) 23:45-50; highlighted sections at page 48, column 3, to page 49, column 1), not all portions of an RNA molecule are equally exposed, and it is challenging to identify vulnerable sites in target RNAs. Native RNA structure restricts the binding of antisense compounds, and presumably that of siRNAs as well. Thus, it is very difficult to predict what portions of an RNA molecule will be accessible *in vivo*, and effective antisense molecules must be found empirically by screening a large number of candidates for their ability to act inside cells. A copy of Branch is submitted herewith for the Examiner's convenience. In view of these facts, one of ordinary skill in the art would not have been motivated to target the region of Survivin mRNA targeted by the antisense compounds of the Bennett et al. patents with an siRNA, and would not have expected to obtain the superior results shown in the present application for such an siRNA.

Regarding the secondary references, none of Tuschl et al., Vickers et al., Morrissey et al., or Arnold et al., either alone or in combination, remedies the deficiencies of the Bennett et al. patents.

Finally, in view of the results obtained with the siRNA of SEQ ID NO:81 in the present application, and contrary to the assertion in the rejection at page 6 of the outstanding Office Action, there was no reasonable expectation of success in designing an siRNA having high inhibitory activity that targets the same region as the antisense compounds disclosed by the Bennett et al. patents in view of the poor inhibitory activity of the latter.

Summary and Conclusions

In view of all the foregoing facts, Applicants respectfully submit that one of ordinary skill in the art would not have been motivated to use an siRNA to target the specific region of Survivin mRNA targeted by the antisense compounds disclosed in the Bennett et al. patents in view of the poor inhibitory activity of the latter. Furthermore, one would not have expected to achieve the high level of inhibition of Survivin expression obtained with the siRNA of SEQ ID NO:81 as in the present application. The Bennett et al. patents provide no reasonable expectation of success with respect to the present invention, and the results obtained with the siRNA of SEQ ID NO:81 are superior and unexpected, obviating *prima facie* obviousness.

Applicants therefore respectfully submit that the present rejection is improper, and should be withdrawn. Such action is requested.

Rejoinder

Applicants note the Examiner's comments at page 5 of the Requirement for Restriction, dated August 13, 2008, that upon a finding of allowability of a product claim, withdrawn process claims that depend from or otherwise include all the limitations of the allowable product claim will be rejoined in accordance with the provisions of 37 C.F.R. 1.104.

Applicants note that method claims 94-96 variously depend either directly or indirectly from elected Group I claims 72-93. Claims 94-96 are therefore eligible for rejoinder upon a finding of allowability of claims 72-93 as presently amended.

Passage to Issue of the present application is believed to be in order, and is respectfully requested.

If the Examiner has any questions, or would like to discuss any matters in connection with this application, he is invited to contact the undersigned at (317) 433-4983.

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December 15, 2008

Attachment:
Highlighted copy of Branch, A., *A good antisense molecule is hard to find*, TIBS (1998) 23:45-50

the folding of the protease sequences when added as separate molecules, both *in vitro* and *in vivo*[15]. One way in which cells change the quantitative properties of proteins is to make allosteric effectors; this method is reversible and requires the continual presence of the effector. Perhaps another method useful in say, terminal differentiation, is the production of separate steric chaperones that irreversibly change the properties of certain specific proteins by influencing their folding.

Acknowledgements

I thank Chris Dobson and Ulrich Hartl for commenting on this article, and Sheena Radford and Nick Price for advice on specific points.

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A good antisense molecule is hard to find

Andrea D. Branch

Antisense molecules and ribozymes capture the imagination with their promise of rational drug design and exquisite specificity. However, they are far more difficult to produce than was originally anticipated, and their ability to eliminate the function of a single gene has never been proven. Furthermore, a wide variety of unexpected non-antisense effects have come to light. Although some of these side effects will almost certainly have clinical value, they make it hard to produce drugs that act primarily through true antisense mechanisms and complicate the use of antisense compounds as research reagents. To minimize unwanted non-antisense effects, investigators are searching for antisense compounds and ribozymes whose target sites are particularly vulnerable to attack. This is a challenging quest.

ANTISENSE STRATEGIES LOOK almost too easy on paper. Simple and elegant schemes can be drawn for both antisense oligodeoxynucleotides (ODNs – short DNA molecules intended to bind to and inhibit target RNAs through complementary Watson–Crick base pairing) and bioengineered ribozymes (catalytic RNA molecules intended to bind and cleave target RNAs). Scientists seek to use these molecules to ablate selected genes and thereby understand their functions, and pharmaceutical developers are working to find nucleic-acid-based therapies. However, the antisense field has been turned on its head by the discovery of 'non-antisense' effects, which occur when a nucleic acid drug acts on some molecule other than its intended target – often through an entirely unexpected mechanism. Non-antisense effects are not necessarily bad. Indeed, some may prove to be a boon to the pharmaceutical industry because they offer an added source of potency. However, their unpredictability confounds research applications of nucleic acid reagents.

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Non-antisense effects are not the only impediments to rational antisense drug design. The internal structures of target RNAs and their associations with cellular proteins create physical barriers, which render most potential binding sites inaccessible to antisense molecules. For Watson–Crick base pairing to occur, nucleic acid drugs must be complementary to exposed regions in their target RNAs and must co-localize with them. When these requirements are met, true antisense effects are enhanced, and unwanted non-antisense effects are minimized. However, optimization is a time-consuming process. Currently, effective nucleic acid drugs must be selected from large pools of candidates. Streamlined approaches for (irrational) *in vitro* selection are needed to speed the discovery of active molecules.

Non-antisense effects: quicksand for some, diamond mines for others

The potential of nucleic acid drugs to deliver 'exquisite specificity'[1] is frequently cited: antisense methods are credited with offering 'the specificity of the genetic code and the versatility of targeting any number of proteins'[2]; and it is said that a therapeutic ribozyme 'can be designed to interact only with its target, and the target is expected to appear only once in the genome, giving one a high degree of assurance that the target – and only that target – has been inhibited'[3]. However, it has never been proven that antisense drugs have the capacity to knock out just one gene, although both ODNs and bioengineered ribozymes can undeniably hit their intended targets[4,5]. The powerful appeal of antisense strategies has been a mixed blessing. The twin concepts that effective antisense reagents are easy to

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design and that they selectively home in on their targets have overshadowed the cautionary messages in articles such as 'Antisense has growing pains'[4], 'Can hammerhead ribozymes be efficient tools to inactivate gene function?'[7], and 'Does antisense exist? (It may, but only under very special circumstances)'[8].

The purpose of this article is to review the factors that make and break specificity in antisense applications and to discuss the need to judge therapeutic compounds and research reagents by separate standards. Only antisense molecules[9–11] and ribozymes[12,13] designed to inhibit RNA targets are considered here, but many of the principles apply to other nucleic acid drugs, such as those used to correct DNA mutations[14], to alter RNA splicing[15], and to control gene expression by forming triple helices with DNA (Ref. 16).

Non-antisense effects pose a dilemma for the pharmaceutical industry[17]. These effects include the stimulation of B-cell proliferation[18] and the inhibition of viral entry into cells[19], responses which are potentially useful. Non-antisense ODNs are already being developed as adjuvants to boost the efficacy of immunotherapies and vaccines[20]. Phase III clinical trials of ISIS 2922 (Ref. 21), a phosphorothioate oligonucleotide (S-ODN) that induces both antisense and non-antisense effects, are also under way in patients with cytomegalovirus-associated retinitis[22]. It is hoped that this compound's diverse mechanisms of action will yield a single drug that provides many of the benefits of combination therapy. However, as Anderson and colleagues have observed, characteristics that are advantages in pharmaceutical drugs can be disadvantages in research reagents[21]. Thus, a safe and effective nucleic acid drug that slows the progression of AIDS would be of tremendous value, even if it were to act by inhibiting a perplexing combination of viral proteins rather than by binding to HIV RNA as originally intended. However, this same compound would be useless as an agent to selectively destroy HIV RNA, and could be ruinous if used in experiments of HIV molecular biology without knowledge of its mechanism of action. Because a single, well-understood mechanism of action cannot be assumed, non-antisense effects create major difficulties for gene hunters. Years of investigation can be required to figure out what an 'antisense' molecule is actually doing, as discussed further below.

Non-antisense effects also have a downside for pharmaceutical developers. Because knowledge of their underlying mechanisms is typically lacking, non-antisense effects muddy the waters. They make true antisense drugs more difficult to design and harder to commercialize. Furthermore, they can be a source of toxicity.

All drugs are dirty: clinical benefit is the pharmaceutical gold standard

Stanley Crooke (Isis Pharmaceuticals) stresses that 'a vast body of experience says that no drug is entirely selective'[23]. Because biologically active compounds generally have a variety of effects, dose–response curves are always needed to establish a compound's primary pharmacological identity. Antisense compounds are no exception. As is true of all pharmaceuticals, the value of a potential antisense drug can only be judged after its intended clinical use is known, and quantitative information about its dose–response curves and therapeutic index is available.

It may be surprising to hear antisense molecules described in the same terms as conventional drugs, but, in fact, nucleic acid drugs should not be thought of as magic bullets. Their therapeutic use will require vigilant monitoring. Compared to the dose–response curves of conventional drugs, which typically span two to three orders of magnitude, those of antisense drugs extend only across a narrow concentration range. Both *in vitro* and *in vivo*, less than a factor of ten often separates the concentration producing no antisense effect from that producing the full antisense effect[22]. Steep dose–response curves commonly indicate that a drug has multiple, synergistic mechanisms of action[24]. A drug with a narrow therapeutic window can be potent and extremely valuable, but can also be tricky to use safely. Since the ratio of antisense to non-antisense effects drops sharply outside a restricted concentration range, it will be challenging to obtain consistent therapeutic results.

Mother Nature's cruel antisense jokes lead to tougher experimental standards

Their powerful allure and favorable press have often caused the problems associated with antisense reagents to be trivialized. In some cases, relaxed standards have been applied. Arthur Krieg (University of Iowa) provided insight into the need for stricter quality control when he shared the results of an informal poll. He reported that 'the estimate that many people have given me of the percentage of accurate published antisense papers ranges from 50% of them being accurate to 5% being accurate'[22].

As discussed previously, when an antisense molecule causes a biological effect, it can be extremely difficult to determine whether the change occurred because the reagent interacted specifically with its target RNA, or because some non-antisense reaction – involving other nucleic acids or proteins – was set in motion[8,25]. When attempting to distinguish between antisense and non-antisense effects, a common strategy has been to use an oligonucleotide in which the sequence of the antisense oligonucleotide is altered. Unfortunately, not all non-antisense effects can be readily detected by this approach, as illustrated by studies of antisense therapies for chronic myeloid leukemia. In this disease, a chromosomal translocation often produces the Philadelphia chromosome, resulting in the synthesis of an oncogenic fusion protein, BCR/ABL. The mRNA for this protein has been regarded as an ideal target for antisense therapies. Several groups have reported inhibition of leukemic cell proliferation by anti-BCR/ABL antisense oligonucleotides. In fact, Vaerman and co-workers cite 16 publications reporting promising findings[26]. However, they discovered that a disappointing, non-antisense mechanism was responsible for their own results, adding weight to studies showing that S-ODNs block proliferation through non-antisense mechanisms (reviewed in Ref. 26). Recent work indicates that cytotoxic ODN breakdown products are responsible for the antiproliferative effects observed[27]. These studies strongly underscore the need to test numerous control ODNs when carrying out antisense research, and to maintain a high index of suspicion.

C. A. Stein (Columbia University) has reviewed many 'non-sequence-specific' (non-antisense) effects caused by S-ODNs, providing dramatic examples of the havoc that has resulted when S-ODNs have unleashed their surfeit of cryptic information. S-ODNs are used because their modified backbones confer nuclease resistance. However, they bind avidly to many proteins, forming complexes with dissociation constants one to three orders of magnitude lower than those of phosphodiester ODNs. In a test of B-cell proliferation and differentiation, S-ODNs were two logs more potent than phosphodiester ODNs of the same sequence[28]. According to Stein, S-ODNs have 'bamboozled' many researchers by inducing biological effects that mimic, and are mistaken for, true and desired antisense effects[8,19].

Addressing the manifest need for stricter experimental standards, Arthur Krieg and C. A. Stein (editors of the journal *Antisense and Nucleic Acid Drug Development*) have published guidelines for designing antisense studies[1]. Recently, the need to use pure oligonucleotide reagents has been stressed. The selective publication of expected (positive) results is being actively discouraged. The confusion that has thus far occurred indicates that each new 'antisense' molecule needs to be tested exhaustively.

How close do current antisense techniques come to single-gene accuracy?

While the ability to knock out a single gene is a luxury in a pharmaceutical compound, specificity is a key feature of a reagent to be used in a research setting. Although single-gene accuracy is not essential for an experimental reagent to be useful, the extraneous perturbations it causes need to be identified. Additionally, as alternative approaches for selective gene ablation (such as the production of genetic knockouts) improve and become easier to carry out, it will be important to know how antisense techniques compare in terms of time, expense and selectivity. This comparison awaits additional information about antisense specificity.

Unfortunately, quantitative data about the magnitude of antisense-induced side reactions are limited. Most of the information is extrapolated from experiments in which the impact of an antisense compound is measured on only a small number of molecules: the intended target RNA, a housekeeping gene, and perhaps a few control RNAs. An antisense molecule is typically taken to be 'specific' if two criteria are met: (1) there is no gross loss of cell viability, and (2) the levels of the target RNA and its associated protein fall much more than those of the control RNAs. However, this type of experimental design is too limited in scope to provide information about global changes in the RNA and protein populations. It does not provide even a rough measure of the signal-to-total noise ratio. Unlike the analysis of Scatchard plots, which allows the interactions between a ligand and a complex mixture of proteins to be explored, this design looks at three or four RNAs and projects the impact on the remaining 10^5 genes. As an additional shortcoming, it provides no direct information about interactions between the antisense molecule and proteins, even though these interactions may lead to the major effects caused by 'antisense' molecules. Because it could provide a before-and-after snap-shot of the protein population, high-resolution two-dimensional gel electrophoresis[29] might shed light on the spectrum of changes induced by antisense molecules. However, a recent round-table discussion suggested that there are no published studies in which this technique has been utilized to evaluate antisense specificity[22].

So far, the concept that an antisense molecule can selectively knock out a single gene appears to have been untested. In the future, several techniques, in addition to two-dimensional gel electrophoresis, might be employed to investigate antisense specificity. For example, as the repository of sequenced genes grows, it will be possible to identify RNAs that contain regions complementary to an antisense molecule and to measure the impact of antisense treatments on these bystander molecules. In addition, broad surveys of mRNA populations could be conducted. To identify changes induced by antisense treatments, RNA from treated and control cells could be reverse-transcribed and the resulting cDNA populations analyzed either by differential display, which separates cDNAs electrophoretically, or by hybridization to gene chips, which are being developed to allow the quantitative monitoring of gene expression patterns[30]. Should unanticipated changes be detected by such surveys, other techniques could be used to distinguish those caused by lack of specificity from those reflecting downstream consequences of the intended antisense reaction. Information about the number of accidental hits and about the nature of the interactions responsible for the changes in the expression of other genes would be useful and would guide future drug development. Today's peak specificity, whatever it is, will almost certainly rise as current strategies are optimized and advances in nucleic acid chemistry bring derivatives with fewer side effects. New compounds are currently under investigation[17,31] and additional derivatives can be expected in the future.

Theoretical limits of specificity

Theoretical calculations provide a useful perspective on antisense specificity. The haploid human genome contains about 3×10^9 bases. In a *random* sequence of this size, any sequence that is 17 nucleotides long or longer would have a high probability of occurring only once – of being unique. To knock out a single gene, an intervention would have to distinguish a 17-base perfect match from one with a single-base mismatch.

In considering whether ODNs have the requisite power of discrimination, it is crucial to know their mechanism(s) of action. These mechanisms may differ from cell type to cell type and may depend upon the exact nature of the target RNA and the ODN. However, there is strong evidence that in several systems, including *Xenopus* oocytes[32] and permeabilized cells[33], the target RNA is destroyed by the action of RNase H. RNase H activities cleave the RNA component of DNA–RNA hybrids. They do not require long hybrid regions as substrates. In fact, *in vitro*, RNase H can cleave a hybrid containing only 4 bp (Ref. 34). In *Xenopus* oocytes, as few as 10 bp are sufficient[35]. For standard ODNs, it is likely that 10 bp are also sufficient in human cells; in the case of certain chemically modified nucleotides, it is proven that as few as 7 bp can lead to cleavage[36]. Random sequences the length of the human genome contain an average of 3000 copies of each 10-nucleotide sequence (10-mer). Thus, it is extremely likely that any particular 10-mer will occur in many RNAs. When an ODN complementary to this 10-mer is introduced into a cell, all of the RNAs containing this 10-mer are at risk for RNase H-mediated cleavage. Of course, not all 3000 copies will be susceptible to cleavage: many will not be present in transcripts, and many that are present in transcripts will be inaccessible. However, if even 1% of the 3000 are hit, 30 genes will be directly affected. Furthermore, the number of 'at risk' sites is probably more than an order of magnitude greater than 3000 for two reasons: (1) ODNs typically contain 20 or more bases, each 20-mer contains 11 10-mers, and each 10-mer would be present 3000 times, on average; and (2) in all likelihood, RNase H does not require 10 consecutive bp for cleavage. Because RNase H requires only a short hybrid region, it is not possible to increase specificity by increasing the length of the ODN. In fact, increasing the length beyond the minimum is likely to have the opposite effect, by stabilizing binding to mismatched sequences, as illustrated in Fig. 1.

Based on studies performed in *Xenopus* oocytes, Woolf and co-workers concluded that it is probably not possible to obtain cleavage of an intended target RNA without also causing at least partial destruction of many non-targeted RNAs (Ref. 35). The ratio of intended to unintended hits will depend on a complex and unpredictable combination of factors that determine whether the antisense molecule and the potential targets co-localize and

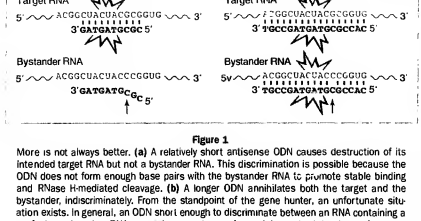

Figure 1

More is not always better. **(a)** A relatively short antisense ODN causes destruction of its intended target RNA but not a bystander RNA. This discrimination is possible because the ODN does not form enough base pairs with the bystander RNA to promote stable binding and RNase H-mediated cleavage. **(b)** A longer ODN annihilates both the target and the bystander, indiscriminately. From the standpoint of the gene hunter, an unfortunate situation exists. In general, an ODN short enough to discriminate between an RNA containing a perfect match and an RNA containing a one-base mismatch is so short that its perfect complement occurs in many different RNAs in a human cell. Thus, although it can distinguish between perfect and imperfect matches, the ODN cannot selectively destroy its target RNA. To overcome this problem, the second generation of ODNs will need special design features to enhance their specificity. In the diagrams, the 'explosion' denotes RNA cleavage by RNase H. ODNs are presented in boldface type, and sequences complementary to all or part of the ODN appear in regular lettering with the remainders of the target and bystander RNAs depicted by wavy lines; black arrows identify a nucleotide mismatch between the bystander RNA and the ODN (the bystander and the target RNA differ at this position).

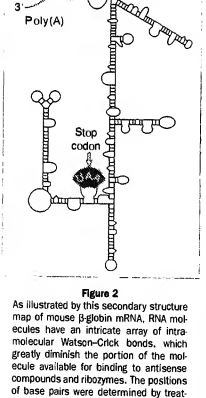

Figure 2

As illustrated by this secondary structure map of mouse β-globin mRNA, RNA molecules have an intricate array of intramolecular Watson–Crick bonds, which greatly diminish the portion of the molecule available for binding to antisense compounds and ribozymes. The positions of base pairs were determined by treating globin mRNA with structure-sensitive nucleases *in vitro*. Redrawn from Ref. 40, with kind permission.

whether the complementary sites in the RNAs are buried under proteins or are involved in intramolecular bonds that make them inaccessible. In the future, even as improvements in antisense chemistry reduce oligonucleotide binding to proteins, the specificity limits imposed by RNase H will remain and will be important to keep in mind when evaluating antisense strategies.

Target site recognition by bioengineered ribozymes is determined by Watson–Crick base pairing and thus has limits of specificity similar to those of ODNs. Ribozymes bind to their target RNAs through a recognition sequence of variable length. Somewhat counterintuitively, a ribozyme with the potential to form a larger number of base pairs with its target RNA does not necessarily have a greater power to discriminate between its intended target and a related bystander RNA than a ribozyme with a shorter recognition sequence. In fact, extending the length of the recognition sequence may reduce a ribozyme's ability to discriminate[37]. It remains to be determined whether there are recognition sequence lengths that are both short enough to allow RNAs that differ from the target at a single nucleotide to be spared cleavage and long enough to allow a unique RNA to be selectively destroyed[38]. It will not be surprising if bioengineered ribozymes are incapable of knocking out single genes, as contemplated by Bertrand and co-workers[7]. Most of these molecules are derived from either hammerhead or hairpin ribozymes[13]. In their natural setting, these ribozymes are covalently attached to their cleavage sites. They self-cleave precursor molecules of subviral (viroid) pathogens[39]. To fulfill their duties, these ribozymes have only to select their target site from the limited number of choices available in the same (small) RNA molecule. Thus, in terms of specificity, bioengineered ribozymes are expected to outperform their natural counterparts. Of course, besides binding to unintended RNAs through Watson–Crick and/or non-Watson–Crick interactions, ribozymes, like other RNAs, are highly charged molecules and have the potential to bind to cellular proteins, thereby producing biologically significant (non-antisense) effects.

As regards the theoretical limits of antisense specificity, it is important to remember that the genome is not a 'random sequence'. Sequences that constitute 'good' antisense targets in one RNA may occur in other RNAs at a higher or lower frequency than random chance would predict. One anecdote reveals how the redundancy of biological sequences could plague antisense methods. A conserved 350-base region at the 5′ end of the hepatitis C virus is considered to be a potential target for antisense drugs. This short region contains a particular 10-mer that is also present in 62 known human mRNAs (Ref. 25), and it contains two 17-mers that occur in known human DNA sequences. Ultimately, the tendency for biological sequences to be reused may limit the specificity of strategies that rely solely on Watson–Crick base pairing for recognition. This tendency will become amenable to detailed analysis soon, as more complete data about human gene sequences become available.

The three As of antisense-mediated gene ablation: access, access and access

Inside cells, it is obviously not possible to improve specificity by raising the temperature or changing the ionic strength, manipulations that are commonly used to reduce background binding in nucleic acid hybridization experiments *in vitro*. Thus, alternative strategies are needed to enhance specificity within cells. One approach has been to deploy multiple antisense compounds, each directed against a different site in the same target RNA and thereby achieve annihilation by molecular triangulation. In addition, successful efforts have been made to exploit the fact that not all

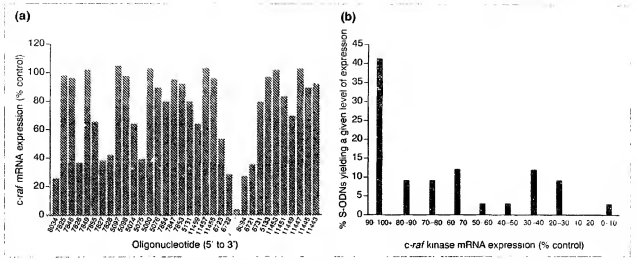

Figure 3

Superior S-ODNs can be found, but they are in the distinct minority. **(a)** Northern hybridization analysis revealed that, of 34 S-ODNs applied to A549 lung carcinoma cells, only one (5132, marked by an asterisk) caused a greater than fivefold reduction in the level of the target, *c-raf* kinase mRNA. Redrawn from Ref. 42, with kind permission. **(b)** Treatment with the majority of the S-ODNs had minimal effect and resulted in levels of the target mRNA that were 50% or more of the level in control cells.

portions of an RNA molecule are equally exposed. If a 10-mer complementary to an antisense ODN occurs in an accessible site in a target RNA and in a protected portion of a bystander, the target will be preferentially destroyed. The challenge is to identify antisense molecules that are complementary to vulnerable sites in target RNAs. This is hard to do. RNAs are complex molecules with intricate internal structures[40], as illustrated by the diagram of β-globin mRNA (Fig. 2).

Recent studies emphasize the extent to which native RNA structure restricts the binding of ODNs. Milner and co-workers[41] tested the ability of 1938 ODNs (ranging in length from monomers to 17-mers) to bind to a 122-nucleotide RNA representing the 5′ end of β-globin mRNA. They found that 'surprisingly few' ODNs bound stably to the mRNA, and concluded that binding is probably 'confined to those regions in the RNA which provide an accessible substructure'[41]. Using short (7 and 8 nucleotides) antisense molecules modified with C-5 propyne pyrimidine and phosphorothioate internucleotide linkages, Wagner and co-workers[36] also determined that the structure of the target RNA is a 'major determinant of specificity'.

Because it is very difficult to predict what portions of an RNA molecule will be accessible *in vivo*, effective antisense molecules must be found empirically by screening a large number of candidates for their ability to act inside cells. Monia and co-workers used northern hybridization to screen 34 20-nt long S-ODNs complementary to *c-raf* kinase and found only one that yielded a greater than fivefold reduction in the target mRNA (Fig. 3a; Ref. 42). Thus, only 3% of the antisense molecules tested in this system were highly effective (Fig. 3b); 40% had almost no effect[42].

Like those of ODNs, ribozyme target sites also vary in their accessibility. Chen and co-workers[43] directly demonstrated that cellular proteins and ribonucleoprotein complexes, such as ribosomes, can prevent ribozyme-mediated cleavage. They showed that a reporter gene was ribozyme-insensitive in wild-type *Escherichia coli* but was ribozyme-sensitive in a 'slow ribosome' mutant. In an accompanying editorial, John Burke (University of Vermont) remarked, 'The simple picture of ribozymes diffusing to, binding, and then cleaving an unstructured RNA is hopelessly oversimplistic'[44].

Rational and irrational design strategies are converging

At any one moment, a combination of the inherent structure of the RNA and its collection of bound proteins limits the number of accessible sites on RNA molecules, thereby providing a basis for specificity. Binding is the rare exception rather than the rule, and antisense molecules are excluded from most complementary sites (see Fig. 4). Since accessibility cannot be predicted, rational design of antisense molecules is not possible. Because design rules are lacking, effective antisense molecules are typically selected from 20–50 candidates in a time-consuming and expensive process that promises to become even more elaborate. If tests of 50 molecules identify good candidates, tests of thousands of compounds should identify better ones. If thousands are to be tested, how should they be designed? Should their sequences be based solely on their potential to form a linear series of Watson–Crick base pairs with the target, or should nucleation sites be included, as they are in naturally occurring antisense RNAs (Ref. 45)? What about non-canonical base-pair interactions, and structural features such stem loops?

The relationship between accessibility to ODN binding *in vitro* and vulnerability to ODN-mediated antisense inhibition *in vivo* is beginning to be explored, and will continue to be an active area of research in the future. It is not yet clear whether *in vitro* screening techniques of the sort used by Milner and co-workers[41] will identify ODNs that are effective *in vivo*. With so many possible sequences to choose from, and the likelihood that *in vitro* studies will not always predict *in vivo* efficacy, straightforward new screening techniques need to be developed for use in cells.

Conclusions

The original concept that ODNs and ribozymes are exquisitely specific and easy to design has been jolted by the discovery of numerous mechanisms of action, leading to non-antisense effects, and the finding that most Watson–Crick binding sites in intended target RNAs

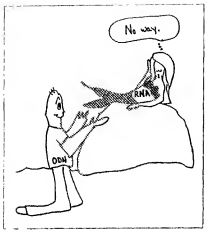

Figure 4
The structure of most potential target sites makes them inaccessible to antisense molecules and ribozymes.

are inaccessible. The time and expense necessary to screen large numbers of potential antisense molecules and ribozymes, and to carefully monitor their *in vivo* effects, raise the stakes for those seeking to use them as genetic probes. Although questions of their ultimate specificity remain, there is growing evidence that antisense molecules can be useful pharmacological tools when applied carefully[17]. In addition, certain non-antisense effects promise to be valuable therapeutically and will be fascinating to investigate. Because non-antisense effects are not currently predictable, rules for rational design cannot be applied to the production of non-antisense drugs. These effects must be explored on a case-by-case basis.

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